(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 018 553 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
30.03.2005 Bulletin 2005/13

(51) Int Cl.[7]: **C12N 15/29**, C12N 15/82, C12N 5/10, C07K 14/415, A01H 5/00

(21) Application number: **99300136.1**

(22) Date of filing: **08.01.1999**

(54) **Transgenic plants with divergent SCaM4 or SCaM5 gene to achieve multiple disease resistance**

Transgene Pflanzen mit divergenten SCaM-4 und SCaM-5 Genen zur Etablierung multipler Krankheitsresistenz

Plantes transgéniques avec les gènes divergents SCaM4 et SCaM5 pour obtenir une résistance aux maladies multiples

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(43) Date of publication of application:
12.07.2000 Bulletin 2000/28

(73) Proprietor: **Korea Kumho Petrochemical Co. Ltd.**
Chongno-Gu, Seoul (KR)

(72) Inventors:
- **Heo, Won-Do**
  Kyungsangnam-do (KR)
- **Cho, Moo-Je**
  Kyungsangnam-do (KR)
- **Song, Pill-Soon**
  Kwangsan-gu, Kwangju-city (KR)
- **Chung, Chang-Ho**
  Seo-gu, Kwangju-city (KR)

(74) Representative: **Benedum, Ulrich Max, Dr. et al**
Haseltine Lake Partners
Motorama Haus 502
Rosenheimer Strasse 30
81669 München (DE)

(56) References cited:
- **LEE S H ET AL: "Identification of a novel divergent calmodulin isoform from soybean which has differential ability to activate calmodulin-dependent enzymes." JOURNAL OF BIOLOGICAL CHEMISTRY, (1995 SEP 15) 270 (37) 21806-12. , XP002109275**
- **HARDING S A ET AL: "Transgenic tobacco expressing a foreign calmodulin gene shows a enhanced production of active oxygen species" EMBO JOURNAL, (17 MAR 1997) VOL. 16, NO. 6, PP. 1137-1144. PUBLISHER: OXFORD UNIV PRESS UNITED KINGDOM, WALTON ST JOURNALS DEPT, OXFORD, ENGLAND OX2 6DP. ISSN: 0261-4189., XP002109276**
- **LEE S. ET AL.: "Differential activation of NAD kinase by plant calmodulin isoforms" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 14, 1997, pages 9252-9259, XP002109277**
- **DOKE N ET AL: "The oxidative burst protects plants against pathogen attack: mechanism and role as an emergency signal for plant bio-defence - a review" GENE, vol. 179, no. 1, 7 November 1996 (1996-11-07), page 45-51 XP004071964 ISSN: 0378-1119**
- **HEO W D ET AL: "Involvement of specific calmodulin isoforms in salicylic acid-independent activation of plant disease resistance responses." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1999 JAN 19) 96 (2) 766-71. , XP002109278**
- **ROBERTS D. ET AL: 'Expression of a calmodulin methylation mutant affects the growth and development of transgenic tobacco plants' PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA vol. 89, September 1992, pages 8394 - 8398**

EP 1 018 553 B1

Description

[0001] The present invention relates to a method for enhancing the multiple disease resistance of higher plants against a wide variety of plant pathogens.

BACKGROUND OF INVENTION

[0002] The present application teaches transgenic plants and plant cells, which have been transformed with the soybean calmodulin isoform genes (SCaM4 and SCaM5) to exhibit greatly enhanced resistance to a wide spectrum of plant pathogens. The present application also teaches an expression vector containing said genes and to a host cell into which said genes in the expression vector have been introduced to plant pathogens-resistant plants. It is also described that transgenic plants expressing a heterologous SCaM 4 or SCaM5 show increased resistance to fungi, bacteria and viruses which normally infect the plants.

[0003] Plants are constantly being challenged by aspiring pathogens. There are great economic losses caused by pathogenic attacks against higher plants in which the natural defenses of plants are inadequate or fail to respond and defend the plants against damage by pathogens. Therefore, control of various plant pathogens is very important in agriculture. Extensive efforts have been focused on controlling pathogenic diseases in crops. However, little success has been achieved by breeding programs to select for crops that are more resistant to pathogens. Furthermore, successful pathogen invasions and diseases ensue if the preformed plant defenses are inappropriate, the plant does not detect the pathogens, or the activated defense responses are ineffective.

[0004] It is well known that the resistance of plants to invading pathogens is accompanied by the deployment of a complex array of defense responses (Jackson et al. 1996. Plant Cell 8:1651-1668). When the pathogen carries a specific avirulence (avr) gene and the plant host contains a cognate resistance, the formation of local lesions occurs at the site of infection that results in inhibition of pathogen growth (termed the hypersensitive response; HR) (The Hypersensitive Reaction in Plants to Pathogens: A Resistance Phenomenon. Goodman, R.N. and Novacky, A.J., APS Press, St. Paul. 1994). Therefore, a plant expressing a particular resistance (R) gene is specifically resistant to pathogens expressing the corresponding avr gene. In addition to the hypersensitive response, a secondary defense response can be triggered that renders uninfected parts of the plant resistant to a variety of virulent pathogens (termed systemic acquired resistance; SAR). The interactions between plants and pathogens lead to a series of defense signal transduction events, including oxidative burst, transient Ca^{+2} increase, salicylic acid accumulation, the synthesis of high levels of pathogenesis-related proteins, phytoalexin biosynthesis and defense gene activations. Accumulating evidence implicates the involvement of a Ca^{2+} signal in certain plant defense responses. A Ca^{2+} ion influx is one of the earliest events in challenged cells (Dixon et al. 1994. Annu. Rev. Phytopathol. 32:479-501) and has been shown to be essential for the activation of defense responses such as phytoalexin biosynthesis, induction of defense-related genes, and hypersensitive cell death (Levine et al. 1996. Curr. Biol. 6:427-437).

[0005] Lee et al. describe in Journal of Biological Chemistry, 1995, 270(37), 21806-12 several divergent isoforms of calmodulin from soybean and suggest the generation of transgenic plants with sense and antisense constructs of SCaM-1 and SCaM-4 to investigate the biological function of each isoform *in vivo.* As transgenic plants with ScaM1 or -4 could not be obtained the authors also speculate that "plants with a perturbation of the calmodulin expression level may not be able to survive".

[0006] The examination of the role of calmodulin within the calcium signal system of plants has also been suggested many times in the prior art (see Harding. et al., in EMBO Journal, 1997, 16(6), 1137-1144; Doke et al. in Gene, 1996, 179(1), 45-51; Roberts et al. in PNAS, USA, 1992, 89, 8394-8398).

[0007] However, direct evidences for the involvement ofCaM in plant disease resistance responses have been unavailable.

[0008] Various approaches have been utilized for attempting to control deleterious fungi, bacteria, viruses and even nematodes. One approach is the application of certain naturally occurring bacteria which inhibit or interfere with fungi or nematodes. Another approach is breeding for resistance, which is primarily focused on the manipulation of minor resistance genes which make small quantitative contributions to the overall resistance of the plant. However, there is often an inability of the plants to recognize the pathogen to cause the defenses of the plants to be induced. Furthermore, the protection provided by these approaches is much narrower than that rendered by full-fledged systemic acquired resistance, and the degree of resistance is much less significant

[0009] Transgenic plants as disclosed hereinafter contain similar levels of specific divergent SCaM4 or SCaM5 protein (0.3-0.5μg/mg total soluble leaf protein) to that of the highly conserved CaM isoforms (SCaM1, SCaM2, and SCaM3) in wild type plants. Upon challenge with pathogens, it has been found that the transgenic plants provided by the method of this invention show long-lasting, broad-spectrum resistance against a variety of pathogens (fungal, viral and bacterial pathogens), similar to systemic acquired resistance. To our knowledge, this is the first *in vivo* evidence not only for the functional differences among CaM isoforms but also for a central role of SCaM4 and SCaM5 for a broad

spectrum of pathogen (including virus, bacteria and fungi) resistance in plants.

SUMMARY OF INVENTION

[0010] The above-stated objects are achieved by a method for enhancing multiple disease resistance of a higher plant as disclosed in claim 1. Preferred embodiments of the invention are described in dependent claims 2 to 8.

[0011] Provided by the method of this invention are higher plant cells transformed with the SCaM4 or SCaM5 gene, resulting in enhanced multiple-resistance to pathogenic attack by one or more plant pathogens. Provided also are the expression vectors accessing SCaM4 or SCaM5 gene which can be able to transform the cells of higher plants according to the present invention.

BRIEF DESCRIPTION OF FIGURES

[0012]

Fig. 1. Schematc diagram of the plant expression vector of pLES9804 harboring an exogenous transgene encoding SCaM4 protein.

Fig. 2. Schematc diagram of the plant expression vector of pLES9805 harboring an exogenous transgene encoding SCaM5 protein.

FIG. 3. Rapid induction of *SCaM*4 and *SCaM*5 during plant defense response. (*A*) Effect of a fungal elicitor on the expression of soybean calmodulin (SCaM) genes. Soybean cell suspension culture (SB-P) was treated with fungal elicitor prepared from *Fusarium solani,* total RNA was isolated at the indicated times, and analyzed for *SCaM*1, *SCaM*4, *SCaM*5, phenylalanine ammonia-lyase (PAL), and β-tubulin mRNA. (*B*) Changes in SCaM protein levels upon fungal elicitor treatment. SB-P cells were treated as described in (*A*) and relative protein levels of SCaM4/5 or SCaM1/2/3 were examined by immunoblot analysis using either anti-SCaM4 or anti-SCaM1 antibody, respectively (16). Relative protein levels of SCaM isoforms were calculated by comparing band intensities and areas in autoradiograms with those of known quantities of standard SCaM1 or SCaM4 proteins using scanning densitometry. (*C*) Effect of various defense signaling molecules on the expression of SCaM genes. SB-P cells were treated for 1 h as indicated above the lanes and the mRNA levels of the SCaM genes were examined by Northern blot analysis. (dH_2O) water control, (Psg) *Pseudomonas syringae* pv. *glycinea* carrying *avr*C, (FE) fungal elicitor prepared from *Phytophihora parasitica* var. *nicotianae,* (FE+CHX) fungal elicitor plus 1 μg/ml cycloheximide, (FE+BAPTA) fungal elicitor plus 5 mM 1,2-*bis*-(*o*-aminophenoxy)-ethane-N,N,N',N-tetraacetic acid, (Ca^{2+}+A23187) 25 μM Ca^{2+} ionophore A23187 plus 5 mM $CaCl_2$, (H_2O_2)2 mM hydrogen peroxide, (G-GO) glucose and glucose oxidase system, (X-XO) xanthine and xanthine oxidase system, (SA) 2 mM salicylic acid, (JA) 100 μM jasmonic acid, (ABA) 100 μM abscisic acid.

FIG. 4. Phenotypes of transgenic tobacco plants constitutively expressing SCaM4 or SCaM5. (*A*) Developmentally regulated formation of spontaneous disease lesion-like necrotic regions in the transgenic plants. The whole plant morphology of an 8-week-old representative transgenic SCaM4 plant (*Right*) which shows that lesions appeared only in the older leaves near bottom of the plant. A normal wild-type plant of similar age is shown on the *Left.* (*B*) A closer look at the spot-like spontaneous lesions formed on the leaf of a 10-week-old SCaM5 transgenic plant (*Right*). A normal leaf of a similar age is shown on the *Left* for comparison. (*C* and *D*) Accumulation of UV-excitable fluorescent material in the cell walls of spontaneously formed lesions. Microscopic examination of a spontaneous lesion under differential interference contrast (*C*) and epifluorecence (*D*) optics after nuclear staining of leaf tissues with 4', 6-diamidino-2-phenylindole.

FIG. 5. Constitutive expression of PR protein genes in transgenic SCaM4 and SCaM5 tobacco plants. (*A*) Immunoblots showing elevated SCaM4 and SCaM5 protein levels in transgenic plants. Total soluble protein (50 μg) from three independent transgenic plant lines was analyzed for SCaM protein levels using either the anti-SCaM4 or anti-SCaM1 antibody. (*B*) Constitutive expression of PR protein genes in the transgenic plants. Total RNA was isolated from a wild type (WT), a control transgenic plant harboring an empty vector (CT), and three representative independent transgenic plants expressing SCaM4 (S4TG) or SCaM5 (S5TG) and examined for the mRNA levels of tobacco SAR genes (31). # indicates numbers of transgenic plant lines. (*C*) Expression of PR protein genes in transgenic SCaM4 and SCaM5 plants in the absence of lesions. RNA blot analysis of *PRI* gene expression in healthy lesion-negative or lesion-positive leaves of the transgenic plants (indicated above lanes as - or +, respectively). (*D*) SA-independent PR protein gene expression. The effect of constitutive expressing SCaM4 or SCaM5 in wild-type plants or *nahG* transgenic plants (33) on PR protein gene expression was examined by RNA gel blot analysis using PR1a and PR5 probes. Data shown are representative results obtained from at least 10 respective independent transgenic plant lines.

FIG. 6. Enhanced disease resistance of transgenic tobacco plants constitutively expressing SCaM4 or SCaM5.

(*A*) Disease responses to the virulent fungal pathogen, *Phytophthora parasitica* var. *nicotianae.* At 7 days after inoculation, plants were examined for disease symptoms. Representative results of wild-type (WT) and transgenic plants expressing SCaM4 (S4TG) or SCaM5 (SSTG) are shown. (*B* and *C*) Fluorescence micrographs of leaves infected with the virulent *P. parasitica* var. *nicotianae.* Infected leaves were cleared, stained with aniline blue, and examined under an ultraviolet-light epifluorescence microscope. In leaves of the wild-type plants (*B*) the spreading of fungal hyphae is evident, but leaves of the transgenic plants (*C*) show the accumulation of UV-excitable fluorescent material in the cells surrounding the fungal penetration sites without appreciable growth of fungal hyphae. Scale bars represent 100 μm. (*D*) *In planta* bacterial growth. *Pseudomonas syringae* pv. *tabacci* was inoculated into leaves of mature wild-type (WT) and transgenic SCaM-4 (S4TG) and SCaM5 (S5 TG) plants at 10^5 cfu/ml, and *in planta* bacterial growth was monitored over 5 days. Data points represent means of two determinations from five independent lines. (E) Elevated resistance of SCaM4 and SCaM5 transgenic plants to the avirulent pathogen, TMV. The second or third fully expanded young leaves from the uppermost part of plants were inoculated with TMV by gently rubbing leaves with carborundum and 2 μg/leaf TMV, and the development of HR lesions were monitored over five days. Data shown are the numbers of HR lesions formed in these plants. In all of these pathogen tests, control transgenic plants transformed with an empty vector showed results essentially similar to those of wild-type plants (data not shown).

DETAILED DESCRIPTION OF THE INVENTION

[0013] Transgenic plants and plant cells, which have been transformed with the soybean calmodulin isoform 4 or 5 (SCaM4 or SCaM5) gene, exhibit greatly enhanced resistance to a wide spectrum of plant pathogens The present description also teaches on expression vector containing said genes and a host cell into which said genes in the expression vector have been introduced to plant pathogens-resistant plants. Transgenic plants expressing a heterologous SCaM show increased resistance to fungi, bacteria and viruses which normally infect the plants.

[0014] In an illustrative preferred embodiment of the invention, cells of tobacco plants are transformed with the divergent SCaM4 or SCaM5 gene. The transformed tobacco plants show enhanced multiple disease resistance against a variety of pathogenic attacks (fungi, viruses and bacteria). The increased multiple disease resistance will allow a higher crop yield and a reduction in the amount of fungicides applied to control decay, allowing for the marketing of more competitive agricultural products.

[0015] SCaM4 and SCaM5 genes are divergent calmodulin isoform from soybean which has differential ability to activate calmodulin-dependent enzymes (Lee et al. 1995. J. Biol. Chem. 270:21806-21812). SCaM5 exhibits -78 % identity of nucleotide sequence with SCaM4.

[0016] A transgenic plant as described herein refers to a plant or plant material that contains one or more inheritable recombinant nucleic acid expression cassettes encoding at least SCaM4 or SCaM5. Preferably, the transgenic, tobacco plant contains at least either SCaM4 or SCaM5 or both

[0017] As used herein, an "overexpressed" SCaM refers to a protein that is produced in higher amount than are produced endogenously. Overexpression can be achieved, for example, by linking a transgene to an appropriate constitutive promoter, such that the transgene is continually expressed. Alternatively, the transgene can be linked to a strong, inducible promoter so that overexpresion can occur on demand. Suitable levels of the overexpression include the expression of trasngene at least about 10-fold over the naturally occurring level of expression of the endogenous transgene being especially preferred. Constitutive promoters suitable for use in the practice of the present invention are widely available and are well known in the art, including the cauliflower mosaic virus 35S (CaMV35S) promoter (U. S. Patent No. 5,097,925) and the like.

[0018] The SCaM4 and SCaM5 as described herein are encoded by recombinant transgene molecules. As used herein, the term "transgene" refers to a DNA or RNA molecule. Transgenes employed herein encode a biologically active amino acid sequence (i.e., protein).

[0019] Transgenes encoding the SCaM4 or SCaM5 protein are typically contained in the expression cassette. The expression cassette refers to a DNA molecule that is able to direct the transcription and translation of a structural gene (i.e., cDNA) so that a desired protein is synthesized. The expression cassette comprises at least one promoter operatively linked to at least one transgene encoding a desired protein, and a transcription terminator sequence. Thus, the protein-encoding segment is transcribed under regulation of the promoter region, into a transcript capable of providing, upon translation, the desired protein. Appropriate reading frame positioning and orientation of the various segments of the expression cassette are within the knowledge of persons of ordinary skill in the art.

[0020] As used herein the term "plasmid" or "vector" refers to circular, double-stranded DNA loops, which are not bound to the chromosome. A plasmid contains DNA capable of causing the expression of DNA sequences contained therein, where such sequences are in operational association with other sequences capable of effecting their expression, such as promoter sequences. Presently preferred vectors for producing invention transgenic tobacco plants are the plasmids pLES9804 and pLES9805, described hereinafter in the EXAMPLES section.

[0021] The term "multiple disease resistance", when used in the context of comparing the level of resistance between an invention transgenic plant and another plant, refers to the ability of the invention transgenic plant to maintain a desirable phenotype in the face of attack, relative to a non-transgenic plant or a single-gene transgenic plant. The level of resistance can be determined by comparing the physical characteristics of the invention plant to non-transgenic plants that either have or have not been exposed to pathogenic infection.

[0022] Methods of introducing the constructs employed herein into suitable host cells, as well as methods applicable for culturing said cells containing a gene encoding a heterologous protein, are generally known in the art. According to the invention, the vector is introduced into the host cell by *Agrobacterium tumefaciens* EHA101. In addition to plant transformation vectors derived from Agrobacterium, high velocity ballistic penetration can be used to insert the DNA constructs of this invention into plant cells. Yet another method of introduction is the fusion of protoplasts with other entities. The DNA may also be introduced into the plant cells by the electroporation.

[0023] In one embodiment of the present invention, two expression cassettes containing transgenes encoding SCaM4 or SCaM5, respectively, are prepared as described in the EXAMPLE. The expression cassettes are combined into a single expression vector, to form a DNA construct, which comprises two individual genes encoding SCaM4 or SCaM5. The vector is then inserted into *A. tumefaciens* cells which contain a disarmed Ti plasmid.

[0024] After transformation, transformed plant cells or plants comprising the said DNA constructs can be identified by employing a selectable marker. Transformed plant cells can be selected by growing the cells in growth medium containing an appropriate antibiotics.

[0025] After selecting the transformed cells, one can confirm expression of the desired heterologous gene. Simple detection of mRNA encoded by the inserted DNA can be achieved by Northern blot hybridization. The inserted sequence can be identified by Southern blot hybridization, as well. Once the presence of the desired transgenes is confirmed, whole plant regeneration can be achieved. All plants which can be regenerated from cultured cells or tissues can be transformed by the present invention.

[0026] Two separate transgenic plants that contain expression cassettes having at least one transgene can be sexually crossed using cross-pollination method to produce a transgenic plant of the present invention. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

[0027] Plants contemplated for use in the practice of the present invention include both monocotyledons and dicotyledons. Exemplary monocotyledons contemplated for use in the practice of the present invention include rice, wheat, maize, barley as well as other grains. Exemplary dicotyledons include tobacco, tomato, potato, soybean, and the like.

[0028] The invention will then be described in greater detail by reference to the following non-limiting examples.

RESULTS

[0029] SCaM-4 is 1,429 bp long and has 664 bp of 5' untranslated region, 450 bp of protein coding sequences, and 315 bp of 3' untranslated region. Protein coding region is composed of 149 amino acids. The polyadenylation signal, ATTAAA was shown in 3' untranslated region.

[0030] . Nucleotide sequence comparison to other plants CaMs, SCaM-1, 2, 3 and bovine CaM reveals more than 70% sequence homology within their protein coding regions. However, the 5' and 3' untranslated regions of SCaM-4 showed no significant homology to other SCaMs, indicating that this cDNA clone is derived from different gene transcripts in soybean genome. In case of actin gene, nucleotide sequence divergency is only 11 to 15 % between plant and non-plant actin, and 6 to 9 % within soybean actin gene family members. SCaM-4 has 30 % divergency compared to SCaM-1, 2, 3 that is surprisingly high.

[0031] Deduced amino acid sequence comparison of SCaM-4 with higher plants CaM and bovine CaM revealed that SCaM-4 shares more than 80 % sequence identity. Otherwise, plant CaMs share more than 95 % sequence identity with each others. Interestingly, the deduced amino acid sequences of SCaM-4 is highly diverged. SCaM-4 is exchanged with SCaM-1,2,3 by 32 amino acids, potato CaM by 26 amino acids, and bovine CaM by 28 amino acids.

[0032] The noticeable exchange is Phe to Tyr in the 3rd Ca^{2+} -binding domain. In bovine CaM, the Tyr residue thought be phosphorylation that functions allowing it to react with target proteins in the absence of Ca^{2+}. The second noticeable exchange is positive charged Lys to negative charged Glu in the 1 st Ca^{2+}-binding domain, and Pro to Asp in the 2nd Ca^{2+}-binding domain. The exchange of Ca^{2+}-binding domain inferred that Ca^{2+}-binding affinity and conformational changes are affected. Further noticeable exchange is Gly^{41} to Asp and Ser^{81} to Ala. This residue is essential to helix bending sites, to allow clasping of the two lobes on to the large variety of basic amphiphilic α-helices in the various targets. Gly^{41} is highly conserved through the evolution presumably because its small size makes the sharp bend possible. The bulkier and charged Asp should interfere with this bend. In addition, the replace Val-145 with Met that three Met, short and unbranched, in the hydrophobic patches may provide the individual contact for the activation of specific targets.

[0033] SCaM-5 consists of 68 bp of 5' untranslated region, 450 bp of open reading frame, and 397 bp of 3' untranslated region. Protein coding region is composed of 149 amino acids. The polyadenylation signal was not found.

[0034] Nucleotide sequence of other plants CaMs, SCaM-1, -2, -3, -4 and bovine CaM reveals more than 70 % sequence homology within their protein coding regions. However, the 5' and 3' untranslated regions of SCaM-5 showed no significant homology to other SCaMs, indicating that this cDNA clone is derived from different gene transcripts in soybean genome. SCaM-5 has an interesting sequence motif of ATTTA in the 3' untranslated region. This motif was known as mRNA destabilizing motif in eukaryote which was recognized by RNase or other cellular factors.

[0035] Deduced amino acid sequence comparison of SCaM-5 with higher plant CaMs and bovine CaM revealed that SCaM-5 share more than 80 % sequence identity with SCaM-4. Interestingly, the deduced amino acid sequences of SCaM-5 are highly diverged from other SCaMs and plant CaMs. SCaM-5 has 18 amino acid substitutions to SCaM-4 and is the most divergent SCaM isoform. These exchanges include five Asp to Glu exchanges as found in the case of SCaM-1 and -2. One of the noticeable exchanges of amino acid residues in SCaM-4 and -5 is the Tyr^{99} residue found in the 3rd Ca^{2+}-binding domain. The Tyr^{99} residue at the 3rd Ca^{2+}- binding domain is found only in animal CaM and not yet found in plant CaM sequences. The Tyr^{99} residue has been thought to be a candidate for phophorylation target by *src* kinase and insulin receptor kinase. Furthermore the Ser^{81} residue which is conserved in all identified CaM sequences is substituted to Ala or Glu in SCaM-4 and -5, respectively. The Ser^{81} residue is observed to be phosphorylated by casein kinase II *in vitro.* As clearly indicated from the sequence comparisons, SCaM-5 can be classified into a new type CaM group together with SCaM-4.

[0036] Treatment of soybean cell suspension cultures with a non-specific fungal elicitor prepared from *Fusarium solani* resulted in a dramatic rise (>10 fold) in mRNA encoded by *SCaM*4 and *SCaM*5 (Fig. 1A), the two *SCaM* genes whose sequences are most divergent from other CaM genes and subsequently will be referred to as the divergent CaM. Their mRNA levels peaked at 1 h and then slowly declined to basal levels by 12 h. The basal expression levels of these two *SCaM* genes in untreated cells were low in comparison to those of the highly conserved *SCaM* genes, *SCaM*1, 2, and 3. In contrast to *SCaM*4 and *SCaM*5, expression of these three conserved SCaM genes was not activated by the elicitor treatment (Fig. I A). Consistent with the changes in SCaM mRNA levels, the protein levels of SCaM4/5 but not SCaM1/2/3 also increased within 30 min of the treatment (Fig. 1B). SCaM4/5 protein levels reached their maximum of approximately 0.5 μg per mg of soluble proteins after 2 h and then slowly declined after 12 h. In contrast, SCaM1/2/3 protein levels were not changed significantly by the fungal elicitor treatment. Interestingly, the induction of the *SCaM*4 and *SCaM*5 genes expression preceded that of the phenylalanine ammonia-lyase gene whose mRNA level began to increase 3 h after treatment (see Fig. 1A). An elicitor prepared from *Phytophthora parasitica* var. *nicotianae* had a similar effect on the expression of these CaM genes (see Fig. 1C).

[0037] We next examined the effect of several potential inducers of plant defense-related genes to gain further insight into the molecular signals involved in the induction of *SCaM*4 and *SCaM*5 gene expression. Both *P. parasitica* elicitor and a bacterial pathogen, *Pseudomonas syringae* pv. *glycinea* (Psg) carrying *avrC,* effectively induced *SCaM*4 and *SCaM*5 gene expression (Fig. 1C). Cycloheximide, a protein synthesis inhibitor, did not block *SCaM*4 and *SCaM*5 induction by the P. *parasitica* elicitor. In contrast, addition of a Ca^{2+}-chelator, 1,2-*bis*-(*o*-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA), abolished this induction of *SCaM4* and *SCaM5,* whereas the Ca^{2+}-ionophore,A23187, alone was sufficient to induce *SCaM*4 and *SCaM*5 expression as effectively as the fungal elicitor. However, application of exogenous salicylic acid (SA; 1 mM to 5 mM) or hydrogen peroxide (H_2O_2; 1 mM to 10 mM) did not induce the expression of *SCaM*4 and *SCaM*5 genes during a 24 h time course (data not shown). A H_2O_2-generating system [glucose and glucose oxidase (Levine, A. et al. 1994. Cell 79:583-593)] and a superoxide-generating system [xanthine and xanthine oxidase (Jabs, T. et al. 1996. Science 273:1853-1856)] also failed to induce *SCaM*4 and *SCaM*5 gene expression. Two unrelated signal molecules, jasmonic acid (JA) (Bergey, D.R. et al. 1996. Proc. Natl. Acad. Sci. USA 93:12053-12508) and abscisic acid (ABA) (Giraudat, J. et al. 1994. Plant Mol. Biol. 26:1557-1578), also were unable to induce *SCaM*4 and *SCaM*5. These results indicate that transcriptional activation of *SCaM*4 and *SCaM*5 genes upon elicitation is a very rapid process which does not require protein synthesis and either precedes synthesis and accumulation of reactive oxygen species (ROS) and SA or is in another disease resistance signaling pathway. Furthermore, their activation was specific; signaling compounds associated with other stress responses such as wounding (JA) and drought (ABA) did not induce *SCaM*4 and *SCaM*5: However, their induction appears to be mediated by an increase of intracellular Ca^{2+} concentration.

[0038] In order to examine the biological functions of these transiently induced, divergent SCaM isoforms in plant defense responses, we constructed transgenic tobacco plants that constitutively expressed SCaM4 or SCaM5 under the control of the constitutive cauliflower mosaic virus 35S promoter. Transgenic plant lines expressing SCaM4 or SCaM5 were first selected by northern analysis and verified by immunoblot analysis (Lee, S.H. et al. 1995. J. Biol. Chem. 270:21806-21812). Interestingly, the transgenic SCaM4 and SCaM5 plants often spontaneously formed disease-like necrotic spots on their leaves (Fig. 2, A and B). The lesions appeared first in the oldest mature leaves, while the top three to four young leaves never developed lesions. These results suggest that spontaneous lesion formation in the transgenic plants was developmentally regulated. Untransformed wild-type and empty vector-transformed control transgenic plants grown under identical conditions did not show these symptoms. To determine whether these lesions were HR-like lesions, they were examined with a fluorescence microscope. HR-like lesions accumulate fluorescent

material in the cell walls that is readily visible under ultraviolet (UV) illumination (Greenberg, J.T. et al. 1994. Cell 77: 551-564), while necrotic regions generated by mechanical wounding or freezing and thawing have no such fluorescence (Mittler, R. et al. 1995. Plant Cell 7:29-42). Leaves of the transgenic plants exhibited accumulation of bright UV-excitable fluorescent material in their lesions, suggesting that these necrotic lesions resemble HR-like lesions (Fig. 2, C and D).

[0039] The constitutive expression of these divergent CaM isoforms SCaM4 and SCaM5 did not affect the level of the highly conserved endogenous tobacco CaM recognized by the anti-SCaM1 antibody (Fig. 3A). The anti-SCaM1 antibody recognized equally the highly conserved CaM isoforms SCaM1, SCaM2, and SCaM3 protein but not the divergent SCaM4 and SCaM5. The anti-SCaM4 antibody cross-reacted with SCaM5 but not with the highly conserved CaM isoforms (16, data not shown). The amounts of SCaM4 and SCaM5 protein in these transgenic plants were estimated to be 0.3-0.5 μg per mg total soluble leaf protein, which is similar to the maximal level of the SCaM4/5 protein induced by a fungal elicitor in soybean cells (see Fig. 1B). Furthermore, the levels of SCaM4 and SCaM5 in transgenic plants did not exceed the protein levels of the highly conserved CaM isoforms (SCaM1, SCaM2, and SCaM3). Thus, it is likely that the phenotypes of these transgenic plants is not due to high level overexpression of SCaM4 or SCaM5, which could lead to abnormal cellular responses (Durner, J. et al. 1997. Trends Plant Sci. 2:266-274), but rather is due to levels of these divergent CaM isoforms which is similar to those after activation of these genes by pathogen attacks.

[0040] Spontaneous lesion forming mutants often show elevated expression of genes encoding pathogenesis-related (PR) proteins and increased resistance to pathogens (Durner, J. et al. 1997. Trends Plant Sci. 2:266-274). Interestingly, in the absence of pathogens, the transgenic SCaM4 and SCaM5 plants expressed at high levels all of the nine SAR marker genes in tobacco that are normally activated during the development of SAR (Ryals, J.A. et al. 1996. Plant Cell 8:1809-1819) (Fig. 3B). Wild-type plants and control empty vector transgenic plants grown under identical conditions did not express these genes. These SAR-associated genes were constitutively expressed throughout the growth and development of the SCaM4 and SCaM5 transgenic plants and were apparent even in the leaves of axenically grown young seedlings which had not developed lesions (Fig. 3C). Thus, expression of PR genes in the transgenic SCaM4 and SCaM5 plants was independent of lesion formation, suggesting that PR gene expression in the transgenic plants is not a consequence of cell death.

[0041] SA is known to be a natural signaling molecule for the activation of certain plant defense responses (Durner, J. et al. 1997. Trends Plant Sci. 2:266-274). The application of exogenous SA to tobacco leaves mimics the pathogen-induced SAR responses such as PR protein synthesis. In addition, endogenous SA levels in several lesion-mimic mutants are substantially higher than those of wild-type plants in the absence of pathogen challenge (Ryals, J.A. et al. 1996. Plant Cell 8:1809-1819). Furthermore, transgenic plants that constitutively expressed the bacterial *nahG* gene, which encodes a SA-degrading enzyme, are defective in their ability to induce SAR (Delaney, T.P. et al. 1994. Science 266:1247-1250). We, therefore, examined whether the disease resistance responses in the SCaM4 or SCaM5 transgenic plants might be due to elevation of endogenous SA levels. Surprisingly, SA levels in SCaM4 and SCaM5 transgenic plants were similar to those of wild-type plants (Table 1). Moreover, infection with tobacco mosaic virus (TMV) resulted in similar increase in SA levels in the transgenic plants and wild-type plants, demonstrating that the expression of SCaM4 and SCaM5 in the transgenic plants did not affect endogenous SA accumulation. Consistent with these observations, the presence and expression of *nahG* gene did not suppress the constitutive expression of PR genes in the SCaM4 and SCaM5 transgenic plants (Fig. 3D). These results strongly argue that SA is not involved in the plant disease resistance responses mediated by SCaM4 or SCaM5.

[0042] Finally, we assessed whether transgenic SCaM4 and SCaM5 plants had altered resistance to pathogens. We used an oomycete fungal pathogen, *Phytophthora parasitica* var. *nicotianae* (the causal agent of black shank disease) to inoculate the transgenic and wild-type plants. At 5 days after *P. parasitica* inoculation, disease symptoms started to appear on the wild-type plants but not on the transgenic plants. By 7 days after inoculation, the wild-type plants had severe disease symptoms such as leaf wilting and stem rot and eventually died by 8 days after inoculation. However, the transgenic plants remained healthy without appreciable disease symptoms (Fig. 4A). The leaves of the fungus-infected wild-type plants showed uninterrupted spreading of fungal hyphae with little callose deposition (Fig. 4B). In contrast, leaves of the inoculated transgenic plants had no such growth of fungal hyphae but instead exhibited bright fluorescence related to callose deposition and accumulation of autofluorescent material in the cells surrounding the fungal penetration sites (Fig. 4C). This is characteristic of an HR. Interestingly, *P. parasitica* var. *nicotianae* is a virulent pathogen on the parental, non-transformed Xanthi-nc tobacco cultivar and does not normally trigger an HR. The transgenic plants also showed enhanced resistance to a virulent bacterial pathogen, *Pseudomonas syringae* pv. *tabacci* (*Pst*). They successfully blocked development of disease symptoms, and the *in planta* growth of *Pst* was retarded greater than 10-fold compared to their growth in wild-type plants (Fig. 4D). In addition, transgenic SCaM4 and SCaM5 plants exhibited increased resistance to a virulent viral pathogen, TMV. The transgenic plants developed TMV-induced HR lesions approximately 12 h earlier than wild-type plants. Furthermore, the transgenic plants had fewer (~5-fold) and smaller TMV-induced lesions (Fig. 4E), which are two characteristics associated with enhanced resistance to TMV.

[0043] In this invention we have demonstrated a central role for the major Ca^{2+} signal transducer, CaM, in plant defense signaling. Our results argue that the divergent CaM isoforms act as both signal receptor and transmitter of

the pathogen-induced Ca^{2+} signal. Divergent CaM isoforms resemble immediate early genes such as fos and jun in animal system in that certain external stimuli immediately activate their expression which then leads to cellular responses (Morgan, J.I. and Curran, T. 1989. Trends Neurosci. 12:459-462). Thus, the divergent CaM isoforms represent novel inducible components of plant defense responses.

[0044] Transgenic plants that constitutively expressed these divergent CaM isoforms had phenotypes similar to those of spontaneous lesion-mimic mutants; however, there are several notable differences. The first concerns the causal relationship between cell death and PR gene expression. While PR gene expression is tightly linked to cell death in *lsd* and *acd* mutants, it is independent of cell death in the SCaM4 and SCaM5 transgenic plants. The second major difference is SA dependence. SA levels in most lesion-mimic mutants are substantially higher than those in normal plants (Dangl, J.L. et al. 1996. Plant Cell 8:1793-1807). In contrast, in the SCaM4 and SCaM5 transgenic plants disease resistance responses were activated without concurrent elevation of endogenous SA level. Furthermore, removal of SA in these transgenic plants by co-expression of the *nahG* gene did not block the constitutive expression of PR genes. These observations strongly suggest that the divergent CaM isoforms activate plant disease resistance responses via a SA-independent pathway(s).

[0045] The present invention provides the first *in vivo* evidence for functional differences among plant CaM isoforms. Only divergent CaM isoforms are induced by pathogens and could trigger defense responses in transgenic plants, whereas the other, highly conserved CaM isoforms such as SCaM1 and SCaM2 did not have these properties (data not shown). The Ca^{2+}/CaM pathway in ROS production is thought to be mediated by the highly conserved CaM isoforms since the divergent CaM isoforms are unable to activate NAD kinase (Lee, S.H. et al. 1997. J. Biol. Chem. 272: 9252-9259). These observations support a model for concerted roles of CaM isoforms in plant defense response against pathogens, in which the highly conserved CaM isoforms mediate ROS increases, while the divergent CaM isoforms activate programmed cell death and defense gene expression.

[0046] Transgenic plants constitutively expressing several other transgenes also have been shown to have lesion-mimic phenotype and altered disease resistance. These genes include *Halobacterium* opsin gene, mutant ubiquitin *ubR48* gene, cholera toxin subunit A1 gene, and yeast invertase (Beffa, R. et al. 1995. EMBO J. 14:5753-5761). These transgenic plants have elevated levels of endogenous SA, which argues that their altered disease resistance responses result from SA accumulation that may be ascribed to the metabolic stress induced by these transgenes (Durner, J. et al. 1997. Trends Plant Sci. 2:266-274). Therefore, it is not clear whether these genes are *bona fide* components of the plant defense response pathway(s). Thus, the divergent CaM isoforms represent one of the first "natural" pathogen-inducible components in plant defense signaling whose constitutive expression leads to enhanced disease resistance. The present invention not only enhance our understanding of the pathway(s) leading to plant disease resistance but also provide new opportunities to genetically engineer plants with resistant to a wide spectrum of pathogens.

EXAMPLES

[0047] The procedures in recombinant DNA technology described below are those well known and commonly employed in the art. These techniques and various other techniques are generally performed according to Sambrook et al., Molecular Cloning - A Laboratory Mannual, (1989). All general references are provided throughout this document. All informations contained herein are incorporated by reference.

EXAMPLE 1 - Isolation of calmodulin cDNAs from soybean

Isolation of SCaM-4 cDNA clone from soybean

[0048] A λZAP (Stratagene) soybean cDNA library, which was constructed from poly $(A)^+$ RNA of half-apical and half-elongating regions of four day-old seedlings, was used for the isolation of SCaM cDNA clones. Library screening was performed by using ECL gene detection system (Amersham) according to the manufacturer's instruction. A rice genomic calmodulin clone, *cam-2,* was used as a probe DNA. Approximately 35,000 phages were plated on *E.coli* XL1-Blue and transferred to nylon membranes (Hybond N+, Amersham). The transferred membrane was denatured by soaking with 0.5 M NaOH, 1.5 M NaCl for 5 min. Then the membrane was neutralized by soaking twice with 0.5 M Tris-HCl (pH 8.0) and 1.5 M NaCl for 3 min period at room temperature. The membrane was alkaline-fixed by soaking with 0.4 N NaOH for 2 min. The membrane prehybridized with hybridization buffer containing 0.5 M NaCl and 5 % blocking agent for 2 hr at 37 °C. Then the membrane was hybridized with horseradish peroxidase-labelled probe DNA for 6 hr at 37 °C with gentle shaking. Posthybridization washes were performed twice in 6 M urea, 0.4 X SSC, 0.5 % SDS at 37 °C for 20 min followed by two washes in 2X SSC for 5 min at room temperate. Then the membrane was detected with detection reagent A, B for 1 min, and then exposed to X-OMAT AR film (Kodak) for 10 min. Positively hybridizing plaques were further purified with subsequent round of plaque hybridization.

[0049] To obtain cDNA insert, purified positive plaques were *in vivo* excised to pBluescript-SK(-) with a helper phage

R408. Two hundred microliters of XL1-Blue host cells ($O.D_{600}$ =1.0) were mixed with 200 µl of λZAP phage stock containing about 1 x 10^6 pfu/ml, and 1 µl of R408 helper phage (1 x 10^6 pfu/ml). After incubation of the mixture at 37 °C for 15 min, 5 ml of 2X YT medium (Bacto tryptone 16g, Bacto Yeast Extract 10g and Sodium chloride 10g each per 1 liter) were added. After incubation for 3 hr at 37 °C with shaking, the mixture was heated at 70 °C for 20 min to inactive the helper phage and to kill bacteria. The mixture was then centrifuged for 10 min at 4000 g and the supernatant was transferred to a sterile tube and stored at 4 °C. To rescue excised phagemid from this stock, 200 µl of XL1-Blue host cells ($O.D_{600}$=1.0) and 200 µl of phage stock were combined, then incubated at 37 °C for 15 min. Then 50 µl of mixture was plated on LB/ampicillin plates followed by incubation at 37 °C for 12 hr. The ampicillin resistant colonies containing rescued phagemid were visible.

[0050] The sequencing reaction for double-stranded plasmid DNA was performed with *Taq* Dye Primer Cycling Sequencing Kit (Applied Biosystems) by using 373A automated DNA sequencer (Applied Biosystems Inc). Nucleotide sequence were analyzed using either DNASIS (Hitachi) on IBM PC or GCG package (Genetics Computer Group, Univ. of Wisconsin) on DECstation 3300/Ultrix system.

[0051] A cDNA encoding soybean CaM was isolated by screening of the λ ZAP cDNA library with a rice genomic clone, *cam-2,* as a probe. From 62 positive clones out of 3.5X10^4 clones, SCaM-1, 2 and 3 were isolated and sequenced previously. Fifteen clones with very low intensity were randomly selected and screened 2 to 3 more times. Throughout the analysis of partial nucleotide sequencing and restriction enzyme mapping of these clones, one clone was selected which contain very long cDNA size and different restriction enzyme map pattern to compare with other SCaMs.

[0052] A finally selected clone was full-length cDNA and designated as SCaM-4 (SEQ. ID No.:1). There are two *Hind*III and one *Eco*RI enzyme sites in internal sequence. Therefore, the *Hind*III fragment, two *Eco*RI - *Hind*III fragments were subcloned to pBluscript SK(-), and *Eco*RI-*Xho*I fragment was performed to self-ligation on pBluscript SK(-). Then, the fragments were sequenced by both orientation. The long *Hind*III fragment was sequenced by deletion sequencing method with *Exo*III enzyme.

Isolation of SCaM-5 cDNA clone from soybean

[0053] A λZAP (Stratagene) soybean cDNA library, which was constructed from poly(A)$^+$ RNA of the half-apical and half-elongating regions of four day-old seedlings, was used for the isolation of CaM cDNA clones. The library was screened by using ECL gene detection system (Amersham) according to the manufacturer's instruction. A rice genomic calmodulin clone, *cam-2,* was used as a probe DNA. Approximately 50,000 phages were plated on *E.coli* XLI-Blue and transferred onto nylon membranes (Hybond N$^+$, Amersham). The transferred membrane was denaturated by soaking with 0.5 M NaOH, 1.5 M NaCl for 7 min. Then the membrane was neutralized by soaking with 0.5 M Tris-HCl (pH 7.5) and 1.5 M NaCl for 7 min at room temperature. The membrane was prehybridized with hybridization buffer containing 0.5 M NaCl and 5 % blocking agent for 2 hr at 37 °C. The membrane was hybridized with a horseradish peroxidase-labelled probe DNA for 12 hr at 37 °C with gentle shaking. Post-hybridization washes were performed twice in 6 M urea, 0.4 x SSC, 0.5 % SDS at 37°C for 20 min followed by two washes in 2 x SSC for 5 min at 37°C. Positively hybridizing plaques were visualized by using the detection reagent A, B for 1 min, and exposed to X-OMAT AR film (Kodak) for 10 min. Positively hybridizing plaques were further purified by subsequent round of plaque hybridization.

[0054] To obtain cDNA inserts, purified positive plaques were *in vivo* excised to pBluescript-SK(-) with a helper phage R408 (Stratagene). Two hundred microliters of XL1-Blue host cells ($O.D_{600}$ =1.0) were mixed with 200 µl of λ ZAP phage stock containing about 1 x 10^6 pfu/ml and 1 µl of helper phage R408. After incubation of the mixture at 37°C for 15 min, 5 ml of 2 x YT medium was added. After incubation for 3 hr at 37 °C with shaking, the mixture was heated at 70 °C for 20 min to inactivate the helper phages and to kill bacteria. The mixture was then centrifuged for 10 min at 4000 g, and the supernatant was transferred to a sterile tube and stored at 4 ° C. To rescue excised phagemid from this stock, 200 µl of XL1-Blue host cells ($O.D_{600}$=1.0) and 100 µl of phage stock were combined, then incubated at 37 °C for 20 min. Then 50 µl of mixture was plated on LB/ampicillin plates followed by incubation at 37 °C for 15 hr. The ampicillin resistant colonies were examined for the presence of appropriate phagemids by alkaline lysis minipreps.

[0055] The sequencing reaction for double-stranded plasmid DNA was performed with a *Taq* Dye Primer Cycling sequencing Kit (Applied Biosystems) by using 373A automated DNA sequencer (Applied Biosystems Inc) as specified by the manufacturer. Nucleotide sequences were analyzed using either a DNASIS (Hitachi Engineering Co.) on IBM PC or GCG package (Genetics Computer Group, Univ. of Wisconsin) on DECstation 3300/Ultrix system (Digital Equipment Corporation).

[0056] A cDNA encoding soybean CaM was isolated by screening of λ ZAP cDNA library with a rice genomic clone, *cam-2,* as a probe. From 62 positive clones out of 3.5 x 10^4 clones, SCaM-1, -2, -3 and -4 were isolated and sequenced previously. But the fifth clone was truncated in 5' region. To obtain a full-length clone, the library was re-screened with a specific probe made from the 3' untranslated region. Throughout the analysis of partial nucleotide sequencing and restriction enzyme mapping of four clones, one clone was selected which has the same nucleotide sequence to the truncated clone. A selected clone was shown to be a full-length cDNA. This clone was designated as a SCaM-5. There

are one *EcoR*I and one *Sac*I enzyme sites in internal sequence.

EXAMPLE 2 --- Construction of Expression Vectors

Construction of plasmid pLES9804

[0057] For the overexpression of SCaM-4 (SEQ. ID No.:1) in tobacco plant, binary vectors were constructed that contained SCaM-4 under the control of the cauliflower mosaic virus 35S subunit (CaMV35S) promoter region and the Agrobacterium nopaline synthase terminator. pGA643 binary vector (An, G. et al. 1988. In Plan Molecular Biology Mannual, eds. Gelvin, S.B. & Schilperoort, R.A., Kluwer Academic, Dordrecht, A3) was digested with *Hpa*I. SCaM-4 cDNA was digested with *Rsa*I. The *Rsa*I-digested 854bp DNA fragment from SCaM-4 cDNA is directly cloned into the *Hpa*I-digested pGA643 vector to give pLES9804. Said vector in *Agrobacterium tumefaciens* EHA101/pLES9804 has been deposited to Korean Collection for Type Culture according to Budapest Treaty (KCTC Accession No. 0545BP).

Construction of plasmid pLES9805

[0058] For the overexpression of SCaM-5 (SEQ. ID NO.:2) in tobacco plant, binary vectors were constructed that contained SCaM-5 under the control of the cauliflower mosaic virus 35S subunit (CaMV35S) promoter resion and the Agrobacterium nopaline synthase terminator. pGA643 binary vector was digested with *Hpa*I. SCaM-5 cDNA was digested with *EcoR*I. Both ends of the DNA fragment (562bp) from SCaM-5 cDNA was converted to blunt ends with an appropriate of Klenow fragment and, by ligation with T4 DNA ligase, cloned into the binary vector to give pLES9805. Said vector in *Agrobacterium tumefaciens* EHA101/pLES9805 has been deposited to Korean Collection for Type Culture according to Budapest Treaty (KCTC Accession No. 0546BP).

EXAMPLE 3 - Generation of Transgenic Tobacco Plants.

[0059] The transgenic tobacco plants expressing SCaM-4 were generated by *Agrobacterium*-medeated leaf disc transformation (Horsch, R.B. et al. 1988. In Plant Molecular Biology Mannual, eds. Gelvin, S.B. & Schilperoort, R.A., Kluwer Academic, Dordrecht, A5). Tobacco leaf discs were prepared from 8-week-old young tobacco plant and soaked in a solution containing MS salts, 3% sucrose, 2 mg/l NAA, 0.5 mg/l BA, and 0.05% MES for 5 min. The leaf discs were inoculated with *Agrobactrium tumefaciens* EHA101 harboring the construct pLES9804 for 2 days. After washing with sterile MS basal medium the leaf discs were transferred onto selection medium (0.5 mg/l BA, MS salts, 3% sucrose, 100 mg/l kanamycin, 250 mg/l sudopen, and 0.8% agar). After 1 month, regenerated shoots were transferrred onto the rooting media containing MS salts, 3% sucrose, 100 mg/l kanamycin, 250 mg/l sudopen and 0.8% agar. Rooted plants were transferred to a mixed bed of vermiculite, perlite, and peat moss(1:1:1) and grown at 25°C with an 16 hrs day-length cycle in a growth chamber. Thirty two regenerated plants were grown. Among them 13 plants expressed SCaM-4 transcripts and SCaM-4 protein.

[0060] The transgenic tobacco plants expressing SCaM-5 were generated by *Agrobacterium*-medeated leaf disc transformation. Tobacco leaf discs were prepared from 8-week-old young tobacco plant and soaked in a solution containing MS salts, 3% sucrose, 2 mg/l NAA, 0.5 mg/l BA, and 0.05% MES for 5 min. The leaf discs were inoculated with *Agrobactrium tumefaciens* harboring the construct pLES9805 for 2 days. After washing with sterile MS basal medium the leaf discs were transferred onto selection medium(0.5 mg/l BA, MS salts, 3% sucrose, 100 mg/l kanamycin, 250 mg/l sudopen, and 0.8% agar). After 1 month, regenerated shoots were transferrred onto the rooting media containing MS salts, 3% sucrose, 100 mg/l kanamycin, 250 mg/l sudopen and 0.8% agar. Rooted plants were transferred to a mixed bed of vermiculite, perlite, and peat moss(1:1:1) and grown at 25°C with an 16 hrs day-length cycle in a growth chamber. Twenty seven regenerated plants were grown on the soil. Among them 19 independent transgenic plant lines expressed SCaM-5 transcripts and proteins.

[0061] R_1 progeny of transgenic plants expressing high level of SCaM4 or SCaM5 was used for the experiments and maintained at 25 °C day and 20 °C night temperature, a 16-h photoperiod, and 65 % relative humidity.

EXAMPLE 4 - Analysis of SCaM expression in transgenic plants

[0062] Immnoblotting-Polyclonal antibodies against two SCaM isoforms, SCaM-1 and SCaM-4, were prepared by immunizing goats subcutanepusly with 10 mg of each purified SCaM protein in the Freund's complete adjuvant. Subsequent boosting injection were done at 3-week intervals with 1 mg of protein in the Freund's incomplete adjuvant. Fifty micrograms of isolated total soluble protein from wild-type and independent transgenic plant lines were electrophoresed on 13.5% SDS polyacrylamide gels. Proteins were transferred onto a PVDF membrane (Millipore) and incubated with either anti-SCaM-1 or anti-SCaM-4 antibody. Protein bands were detected using the ECL system after

incubating with horseradish peroxidase-conjugated antigoat IgG (ICN Biomedicals). The amounts of SCM-4 or SCaM5 protein in the transgenic tobacco plants were estimated to be 0.3 or 0.5 μg per mg total soluble leaf protein, respectively.

[0063] Northern blot hybridization-Total RNA was isolated from a wild-type, a control transgenic plant harboring an empty vector, and three representative independent transgenic plants expressing SCaM-4 or SCaM5. Ten μg of isolated total RNA was separated on denaturing 1.5% agarose formaldehyde gels. Ethidium bromide was included to verify equal loading of RNA. After transfer to Gene Screen membranes, filter were hybridized with ^{32}P-labelled gene-specific probe in Church's buffer at 65° C for 16hrs. SCaM-4 specific probe was cut out from SCaM-4 or SCaM-5 cDNA with *Eco*RI and *Xho*I and was gel purified. After incubating, filters were washed for 20 min with 2 X SSC twice, for 20 min with 1 X SSC twice, for 20 min with 0.5 X SSC once, and then for 20 min with 0.5 X SSC, 0.1% SDS at 60°C once. Filters were exposed to X-ray films at -80°C for 1 hr to 6 days.

EXAMPLE 5 - Analysis of the Disease Resistance of Transgenic Plants.

[0064] The invention transgenic tobacco plants, described above, were subjected to the fungal, bacterial and viral infection assays. The results are shown in Fig. 4. *TMV treatment* - Tobacco plants [*Nicotiana tabacum* cv. Xanthi nc (NN)/SCaM-4 or-5 transgenic] were grown at 25°C in a growth chamber programmed for a 16-hr light cycle. Seven- to eight-week-old tobacco plants were either inoculated by rubbing fully expanded leaves with carborundum plus TMV (U1 strain, 2 μg/ml or as indicated in 50 mM phosphate buffer, pH 7.0) or buffer only (mock). After infection, plants were washed by spraying sterile water to remove the carborundum from the leaves. The plants were maintained at 25°C throughout the infection in a growth chamber. The development of HR lesions were monitored over 5 days. An empty vector transgenic plants were simultaneously tested as a control.

[0065] *Fungus treatment- Phytophthora parasitica* pv. *nicotianae* mycelia were suspended in sterile water and spread-plated on V8 agar plates [200 ml of V8 juice, 3.0 g of $CaCO_3$, and 18 g of agar per liter (pH 6.15)] supplemented with 1 g of dried tobacco leaves per liter and incubated at 25°C in a continuous light. After a mycelial lawn was established (2-4 weeks), small squares (~8 mm^2) were cut and adhered on the agar media (under 4-week-old tobacco plants). They were transferred to an incubation chamber at 25°C with a 16-hr photopered. The development of HR lesions were monitored over 5 days. An empty vector transgenic plants were simultaneously tested as a control.

[0066] *Bacteria treatment- Pseudomonas syringae* pv. *tobaci* (ATCC 11528) were grown at 28°C overnight in King's B medium containing 50 μg/ml rifampicin, washed twice, resuspended, and diluted in 10 mM $MgCl_2$. Small areas of healthy leaves were inoculated by infiltration with a suspension of 10^5 bacterial cells per ml using a 1-ml hypodermic syringe without a needle. One side of each leaf was inoculated as four different places with ~20 μl of bacterial suspension per wound, and the other side was mock-inoculated with 20 μl of 10 mM $MgCl_2$. Leaf discs (6mm diameter) was collected with a puncher. Bacteria were extracted by maceration of leaf discs in 10 mM $MgCl_2$ and serial dilutions were plated on King's B media with 50 μg per ml rifampicin and 10 μg per ml malidixic acid. Colonies were counted at 48 hrs after incubation at 28°C. *In planta* bacterial growth was monitored over 5 days. The development of HR lesions were monitored over 5 days. An empty vector transgenic plants were simultaneously tested as a control.

[0067] The transgenic tobacco plants, which were transformed with pLES9804 or pLES9805, respectively, and ultimately revealed a multiple disease resistance to a wide variety of pathogens, have been deposited to Korean Collection for Type Cultures according to the Budapest Treaty (KCTC Accession No. 0543BP and 0544BP, respectively).

Table 1.

Endogenous salicylic acid (SA) levels in transgenic SCaM-4 and SCaM-5 plants.			
Plants	Uninfected	TMV-infected	
	Free SA	Free SA	Bound SA
Wild type	0.042 ± 0.005	2.8 ± 0.5	2.2 ± 0.5
Transgenic SCaM-4	0.031 ± 0.010	4.1 ± 1.0	2.4 ± 0.8
Transgenic SCaM-5	0.035 ± 0.005	2.5 ± 0.5	1.6 ± 0.5

[0068] The levels of SA (μg/g fresh weight) were determined from the top forth or fifth middle leaves of mature plants as described (Herbers, K. et al. 1996. Plant Cell 8:793-803). Data are the means of two determinations from five independent transgenic plant lines. Bound SA levels were below detection-limit in uninfected plants. Endogenous SA levels from tobacco mosaic virus (TMV)-infected plants were determined 10 days after inoculation.

Complete nucleotide and deduced amino acid sequences of SCaM-4

```
1        ATTTCTTACCTCTGTAGGAAAAATCAAACTCACATACTTACCGGATGAGTCTGTTT
57   CTTACCGAGGACAAGCTTTATAAGTGTCTGAGTCAGTCTTCTGGTGCAAGCAAAGCAACC
117  AGCTAGCGTGGTCTTTGGGTGGGTGGTGTGTGGAAATTGGATATTGATGAGAACGCAGAC
177  TCTACACACCTCCCCATACTCCATACTCCATACCCTTTTCAAGTTTGAATTAATCCCTTC
237  TTGTTTTGTCCCCTAAACACCAAACCCTTCCAGCTGTCAAGATTTTCTCCGTATTATACT
297  AATCAAATTAAATATCATCATACATTAAAAAATCATTCAGCGCATTTAAACCATCGTGCC
357  CCACCCCATTTGTACTATACTACTATGTAACAATTACAATTACAATGTGATTCTTTTTTA
417  CACAATAAAGCCACGAGTATGGCAACAAAATGCAAGTCATATACTTAATTTGACTCGGTA
477  AATTAAAATTTTAAAAGAAATCTTGCAAGACTAGCTAGCTAGCATCACCTAAGTTCTACA
537  ATATAGCCCAGCTAGGATTGAGCTATACTCAACTCAACACATCATTCTCTCTTTCCCTCT
597  CTATCTCTATCTCTCTTTCTCTGTCTCTTTTCTGGTTGAAGTTTGAAAGACAAATACACC

657  ATGGCAGATATCCTGAGTGAAGAACAGATTGTTGATTTTAAAGAGGCCTTTGGCTTGTTT

          M  A  D  I  L  S  E  E  Q  I  V  D  F  K  E  A  F  G  L  F

717  GACAAAGATGGAGATGGTTGCATTACTGTGGAAGAACTTGCCACTGTCATTCGGTCATTG

          D  K  D  G  D  G  C  I  T  V  E  E  L  A  T  V  I  R  S  L

777  GATCAGAACCCCACTGAAGAAGAGCTCCAAGATATGATAAGCGAAGTCGATGCAGATGGC

          D  Q  N  P  T  E  E  E  L  Q  D  M  I  S  E  V  D  A  D  G

837  AATGGAACCATTGAATTTGACGAGTTCTTGAGCTTGATGGCCAAGAAAGTTAAAGACACT

          N  G  T  I  E  F  D  E  F  L  S  L  M  A  K  K  V  K  D  T

897  GATGCAGAGGAGGAGCTCAAAGAAGCTTTCAAGGTTTTTGACAAAGATCAAAATGGCTAC

          D  A  E  E  E  L  K  E  A  F  K  V  F  D  K  D  Q  N  G  Y

957  ATATCAGCTAGTGAGTTGAGACACGTAATGATCAATCTAGGGGAAAAACTAACCGATGAA

          I  S  A  S  E  L  R  H  V  M  I  N  L  G  E  K  L  T  D  E

1017 GAGGTGGAGCAGATGATTAAAGAAGCAGATTTGGACGGTGATGGCCAAGTTAACTATGAG

          E  V  E  Q  M  I  K  E  A  D  L  D  G  D  G  Q  V  N  Y  E

1077 GAATTCGTCAAGATGATGATGACCGTTCGATGAAACACTCTCACCTAATTAATTGGATTG

          E  F  V  K  M  M  M  T  V  R  *

1137 GACACCAATTTGTTAATTCAAAATTCATTGGCTTCCAACCTCCCAATGAAATAAGTGTTC
1197 TTTCTTTATTATTGTTTGTTGTATTGTACTATTATTCTACTTGTACTTAGTAATGACCAA
1257 GCAGTAGATTGGCACCCCCATTCCATTTGATCCATTCCAAAATTAAATTACTATTCTTGT
1317 AATTTTAGTTCAGTACATTTTCTATCCTCCGAGAGTAAGAAACCCAAGGAGCATATCTAC
```

1377 CCATTAATTATGCATGACTTTTACC

Complete nucleotide and deduced amino acid sequences of SCaM-5

```
1                                                                CTCCCTCT
9    CTCTTTTCTAAGTCACAAAATATTGTCTTAGTTTTCATTTGAAGCTCAAACATTAACACC
69   ATGGCAGATGTTCTGAGTGAAGAACAGATTAGTGAGATCAAAGAAGCCTTTGGCTTGTTT

       M  A  D  V  L  S  E  E  Q  I  S  E  I  K  E  A  F  G  L  F

129  GACAAAGATGGTGATGGGTGCATTACTGTGGACGAATTTGTCACGGTTATCCGGTCATTG

       D  K  D  G  D  G  C  I  T  V  D  E  F  V  T  V  I  R  S  L

189  GTTCAGAACCCCACAGAAGAAGAGCTCCAAGACATGATAAACGAGGTAGATGCAGATGGT

       V  Q  N  P  T  E  E  E  L  Q  D  M  I  N  E  V  D  A  D  G

249  AATGGAACCATTGAATTTGTTGAGTTTTTGAACTTAATGGCCAAGAAAATGAAGGAAACT

       N  G  T  I  E  F  V  E  F  L  N  L  M  A  K  K  M  K  E  T

309  GATGAAGAGGAAGATCTCAAAGAGGCTTTCAAGGTGTTTGACAAGGATCAAAATGGCTAC

       D  E  E  E  D  L  K  E  A  F  K  V  F  D  K  D  Q  N  G  Y

369  ATTTCAGCAAGTGAGTTGAGACACGTTATGATCAATCTGGGTGAAAAACTAACTGATGAG

       I  S  A  S  E  L  R  H  V  M  I  N  L  G  E  K  L  T  D  E

429  GAGGTGGAGCAGATGATTGAAGAAGCAGATTTGGATGGTGATGGTCAAGTTAATTATGAT

       E  V  E  Q  M  I  E  E  A  D  L  D  G  D  G  Q  V  N  Y  D

489  GAATTTGTCAAGATGATGATGACTATTGGATGAAATTTTCAAACAACAAATTTATTGTTC

       E  F  V  K  M  M  M  T  I  G  *

549  GAATTCGATCATTTGGGCCCCAATCTCATTCACTCGCATGAAATAATAAGTCTTCCAATC
609  AATTTTTGGTGTATTTTACTTGTAGCACATGATATGAATGACCAATGTATTAGAGTGACA
669  TCTCAGCCAATTTGATTTGTTCAAACTCCAACCCATAATTATTCTTTCAGTTTTAGTTTA
729  GCATTCCTTATGTTTTAAAATTAATTAGAGGGAATTTATTCAAGAACTTCTGAAATTAGA
789  CTCTTTTTTGAAAGATTAATGTATGCTACAATTTATTCTTTCTCTTGTGAACATGGTATT
849  GCTACAATTTATTTATATCATTCAGAAAAATAAAAAACAGCACAAGAATTAAACAAAAAA
909  AAAAAAAA
```

Sequence Listing

[0069]

(2) INFORMATION FOR SEQ. ID No.:1:

(i) SQUENCE CHARACTERISTICS:

(a) LENGTH: base pairs
(b) TYPE: nucleic acid
(c) STRANDEDNESS: both
(d) TYPOLOGY: both

(ii) MOLECULAR TYPE: cDNA (ix) FEATURE:

(a) NAME/KEY: CDS
(b) LOCATION: 1.. 1401
(c) OTHER INFORMATION:/PRODUCT = *G*. *max* calmodulin4 (SCaM4)

(xi) SEQUENCE DESCRIPTION: SEQ. ID No.:1:

```
1          ATTTCTTACCTCTGTAGGAAAAATCAAACTCACATACTTACCGGATGAGTCTGTTT
57    CTTACCGAGGACAAGCTTTATAAGTGTCTGAGTCAGTCTTCTGGTGCAAGCAAAGCAACC
117   AGCTAGCGTGGTCTTTGGGTGGGTGGTGTGTGGAAATTGGATATTGATGAGAACGCAGAC
177   TCTACACACCTCCCCATACTCCATACTCCATACCCTTTTCAAGTTTGAATTAATCCCTTC
237   TTGTTTTGTCCCCTAAACACCAAACCCTTCCAGCTGTCAAGATTTTCTCCGTATTATACT
297   AATCAAATTAAATATCATCATACATTAAAAAATCATTCAGCGCATTTAAACCATCGTGCC
357   CCACCCCATTTGTACTATACTACTATGTAACAATTACAATTACAATGTGATTCTTTTTTA
417   CACAATAAAGCCACGAGTATGGCAACAAAATGCAAGTCATATACTTAATTTGACTCGGTA
477   AATTAAAATTTTAAAAGAAATCTTGCAAGACTAGCTAGCTAGCATCACCTAAGTTCTACA
537   ATATAGCCCAGCTAGGATTGAGCTATACTCAACTCAACACATCATTCTCTCTTTCCCTCT
597   CTATCTCTATCTCTCTTTCTCTGTCTCTTTTCTGGTTGAAGTTTGAAAGACAAATACACC

657   ATGGCAGATATCCTGAGTGAAGAACAGATTGTTGATTTTAAAGAGGCCTTTGGCTTGTTT

       M  A  D  I  L  S  E  E  Q  I  V  D  F  K  E  A  F  G  L  F

717   GACAAAGATGGAGATGGTTGCATTACTGTGGAAGAACTTGCCACTGTCATTCGGTCATTG

       D  K  D  G  D  G  C  I  T  V  E  E  L  A  T  V  I  R  S  L

777   GATCAGAACCCCACTGAAGAAGAGCTCCAAGATATGATAAGCGAAGTCGATGCAGATGGC

       D  Q  N  P  T  E  E  E  L  Q  D  M  I  S  E  V  D  A  D  G

837   AATGGAACCATTGAATTTGACGAGTTCTTGAGCTTGATGGCCAAGAAAGTTAAAGACACT

       N  G  T  I  E  F  D  E  F  L  S  L  M  A  K  K  V  K  D  T

897   GATGCAGAGGAGGAGCTCAAAGAAGCTTTCAAGGTTTTTGACAAAGATCAAAATGGCTAC

       D  A  E  E  E  L  K  E  A  F  K  V  F  D  K  D  Q  N  G  Y

957   ATATCAGCTAGTGAGTTGAGACACGTAATGATCAATCTAGGGGAAAAACTAACCGATGAA

       I  S  A  S  E  L  R  H  V  M  I  N  L  G  E  K  L  T  D  E

1017  GAGGTGGAGCAGATGATTAAAGAAGCAGATTTGGACGGTGATGGCCAAGTTAACTATGAG

       E  V  E  Q  M  I  K  E  A  D  L  D  G  D  G  Q  V  N  Y  E

1077  GAATTCGTCAAGATGATGATGACCGTTCGATGAAACACTCTCACCTAATTAATTGGATTG

       E  F  V  K  M  M  M  T  V  R  *

1137  GACACCAATTTGTTAATTCAAAATTCATTGGCTTCCAACCTCCCAATGAAATAAGTGTTC
1197  TTTCTTTATTATTGTTTGTTGTATTGTACTATTATTCTACTTGTACTTAGTAATGACCAA
1257  GCAGTAGATTGGCACCCCCATTCCATTTGATCCATTCCAAAATTAAATTACTATTCTTGT
1317  AATTTTAGTTCAGTACATTTTCTATCCTCCGAGAGTAAGAAACCCAAGGAGCATATCTAC
1377  CCATTAATTATGCATGACTTTTACC
```

(2) INFORMATION FOR SEQ. ID No.:2:

(i) SQUENCE CHARACTERISTICS:

(a) LENGTH: base pairs
(b) TYPE: nucleic acid
(c) STRANDEDNESS: both
(d) TOPOLOGY: both

(ii) MOLECULAR TYPE: cDNA
(ix) FEATURE:

(a) NAME/KEY: CDS
(b) LOCATION: 1.. 916
(c) OTHER INFORMATION:/PRODUCT = *G. max* calmodulin5 (SCaM5)

(xi) SEQUENCE DESCRIPTION: SEQ. B3 No.:2:

```
1                                                                  CTCCCTCT
9    CTCTTTTCTAAGTCACAAAATATTGTCTTAGTTTTCATTTGAAGCTCAAACATTAACACC
69   ATGGCAGATGTTCTGAGTGAAGAACAGATTAGTGAGATCAAAGAAGCCTTTGGCTTGTTT

       M   A   D   V   L   S   E   E   Q   I   S   E   I   K   E   A   F   G   L   F

129  GACAAAGATGGTGATGGGTGCATTACTGTGGACGAATTTGTCACGGTTATCCGGTCATTG

       D   K   D   G   D   G   C   I   T   V   D   E   F   V   T   V   I   R   S   L

189  GTTCAGAACCCCACAGAAGAAGAGCTCCAAGACATGATAAACGAGGTAGATGCAGATGGT

       V   Q   N   P   T   E   E   E   L   Q   D   M   I   N   E   V   D   A   D   G

249  AATGGAACCATTGAATTTGTTGAGTTTTTGAACTTAATGGCCAAGAAAATGAAGGAAACT

       N   G   T   I   E   F   V   E   F   L   N   L   M   A   K   K   M   K   E   T

309  GATGAAGAGGAAGATCTCAAAGAGGCTTTCAAGGTGTTTGACAAGGATCAAAATGGCTAC

       D   E   E   E   D   L   K   E   A   F   K   V   F   D   K   D   Q   N   G   Y

369  ATTTCAGCAAGTGAGTTGAGACACGTTATGATCAATCTGGGTGAAAAACTAACTGATGAG

       I   S   A   S   E   L   R   H   V   M   I   N   L   G   E   K   L   T   D   E

429  GAGGTGGAGCAGATGATTGAAGAAGCAGATTTGGATGGTGATGGTCAAGTTAATTATGAT

       E   V   E   Q   M   I   E   E   A   D   L   D   G   D   G   Q   V   N   Y   D

489  GAATTTGTCAAGATGATGATGACTATTGGATGAAATTTTCAAACAACAAATTTATTGTTC

       E   F   V   K   M   M   M   T   I   G   *

549  GAATTCGATCATTTGGGCCCCAATCTCATTCACTCGCATGAAATAATAAGTCTTCCAATC
609  AATTTTTGGTGTATTTTACTTGTAGCACATGATATGAATGACCAATGTATTAGAGTGACA
669  TCTCAGCCAATTTGATTTGTTCAAACTCCAACCCATAATTATTCTTTCAGTTTTAGTTTA
729  GCATTCCTTATGTTTTAAAATTAATTAGAGGGAATTTATTCAAGAACTTCTGAAATTAGA


789  CTCTTTTTTGAAAGATTAATGTATGCTACAATTTATTCTTTCTCTTGTGAACATGGTATT
849  GCTACAATTTATTTATATCATTCAGAAAAATAAAAAACAGCACAAGAATTAAACAAAAAA
909  AAAAAAAA
```

SEQUENCE LISTING

[0070]

<110> Korea Kumho Petrochemical., Ltd.

<120> Transgenic plants with divergent SCaM4 or SCaM5 gene to achieve multiple disease resistance

<130> HL71488

<140> 99300136.1
<141> 1999-01-08

<160> 4

<170> PatentIn Ver. 2.0

<210> 1
<211> 1401
<212> DNA
<213> Unknown Organism

<220>
<223> Description of Unknown Organism:lambda ZAP (Stratagene) soybean cDNA library

<220>
<221> CDS
<222> (657)..(1106)

<400> 1

atttcttacc tctgtaggaa aaatcaaact cacatactta ccggatgagt ctgtttctta 60

ccgaggacaa gctttataag tgtctgagtc agtcttctgg tgcaagcaaa gcaaccagct 120

agcgtggtct ttgggtgggt ggtgtgtgga aattggatat tgatgagaac gcagactcta 180

cacacctccc catactccat actccatacc cttttcaagt ttgaattaat cccttcttgt 240

tttgtcccct aaacaccaaa cccttccagc tgtcaagatt ttctccgtat tatactaatc 300

aaattaaata tcatcataca ttaaaaaatc attcagcgca tttaaaccat cgtgccccac 360

cccatttgta ctatactact atgtaacaat tacaattaca atgtgattct tttttacaca 420

ataaagccac gagtatggca acaaaatgca agtcatatac ttaatttgac tcggtaaatt 480

aaaattttaa aagaaatctt gcaagactag ctagctagca tcacctaagt tctacaatat 540

```
agcccagcta ggattgagct atactcaact caacacatca ttctctcttt ccctctctat 600

ctctatctct ctttctctgt ctcttttctg gttgaagttt gaaagacaaa tacacc atg 659
                                                              Met
                                                               1

gca gat atc ctg agt gaa gaa cag att gtt gat ttt aaa gag gcc ttt  707
Ala Asp Ile Leu Ser Glu Glu Gln Ile Val Asp Phe Lys Glu Ala Phe
               5                  10                  15

ggc ttg ttt gac aaa gat gga gat ggt tgc att act gtg gaa gaa ctt  755
Gly Leu Phe Asp Lys Asp Gly Asp Gly Cys Ile Thr Val Glu Glu Leu
         20                  25                  30

gcc act gtc att cgg tca ttg gat cag aac ccc act gaa gaa gag ctc  803
Ala Thr Val Ile Arg Ser Leu Asp Gln Asn Pro Thr Glu Glu Glu Leu
     35                  40                  45

caa gat atg ata agc gaa gtc gat gca gat ggc aat gga acc att gaa  851
Gln Asp Met Ile Ser Glu Val Asp Ala Asp Gly Asn Gly Thr Ile Glu
 50                  55                  60                  65

ttt gac gag ttc ttg agc ttg atg gcc aag aaa gtt aaa gac act gat  899
Phe Asp Glu Phe Leu Ser Leu Met Ala Lys Lys Val Lys Asp Thr Asp
                 70                  75                  80

gca gag gag gag ctc aaa gaa gct ttc aag gtt ttt gac aaa gat caa  947
Ala Glu Glu Glu Leu Lys Glu Ala Phe Lys Val Phe Asp Lys Asp Gln
             85                  90                  95

aat ggc tac ata tca gct agt gag ttg aga cac gta atg atc aat cta  995
Asn Gly Tyr Ile Ser Ala Ser Glu Leu Arg His Val Met Ile Asn Leu
        100                 105                 110

ggg gaa aaa cta acc gat gaa gag gtg gag cag atg att aaa gaa gca 1043
Gly Glu Lys Leu Thr Asp Glu Glu Val Glu Gln Met Ile Lys Glu Ala
    115                 120                 125

gat ttg gac ggt gat ggc caa gtt aac tat gag gaa ttc gtc aag atg 1091
Asp Leu Asp Gly Asp Gly Gln Val Asn Tyr Glu Glu Phe Val Lys Met
130                 135                 140                 145

atg atg acc gtt cga tgaaacactc tcacctaatt aattggattg gacaccaatt 1146
Met Met Thr Val Arg
               150

tgttaattca aaattcattg gcttccaacc tcccaatgaa ataagtgttc tttctttatt 1206
```

attgtttgtt gtattgtact attattctac ttgtacttag taatgaccaa gcagtagatt 1266

ggcaccccca ttccatttga tccattccaa aattaaatta ctattcttgt aattttagtt 1326

cagtacattt tctatcctcc gagagtaaga aacccaagga gcatatctac ccattaatta 1386

tgcatgactt ttacc 1401

<210> 2
<211> 150
<212> PRT
<213> Unknown Organism

<400> 2

Met Ala Asp Ile Leu Ser Glu Glu Gln Ile Val Asp Phe Lys Glu Ala
1 5 10 15

Phe Gly Leu Phe Asp Lys Asp Gly Asp Gly Cys Ile Thr Val Glu Glu
20 25 30

Leu Ala Thr Val Ile Arg Ser Leu Asp Gln Asn Pro Thr Glu Glu Glu
35 40 45

Leu Gln Asp Met Ile Ser Glu Val Asp Ala Asp Gly Asn Gly Thr Ile
50 55 60

Glu Phe Asp Glu Phe Leu Ser Leu Met Ala Lys Lys Val Lys Asp Thr
65 70 75 80

Asp Ala Glu Glu Glu Leu Lys Glu Ala Phe Lys Val Phe Asp Lys Asp
85 90 95

Gln Asn Gly Tyr Ile Ser Ala Ser Glu Leu Arg His Val Met Ile Asn
100 105 110

Leu Gly Glu Lys Leu Thr Asp Glu Glu Val Glu Gln Met Ile Lys Glu
115 120 125

Ala Asp Leu Asp Gly Asp Gly Gln Val Asn Tyr Glu Glu Phe Val Lys
130 135 140

Met Met Met Thr Val Arg
145 150

<210> 3
<211> 916
<212> DNA
<213> Unknown organism

<220>
<223> Description of Unknown Organism:lambda ZAP (Stratagene) soybean cDNA library

<220>
<221> CDS
<222> (69)..(518)

<400> 3

```
ctccctctct cttttctaag tcacaaaata ttgtcttagt tttcatttga agctcaaaca 60

ttaacacc atg gca gat gtt ctg agt gaa gaa cag att agt gag atc aaa 110
         Met Ala Asp Val Leu Ser Glu Glu Gln Ile Ser Glu Ile Lys
           1               5                  10

gaa gcc ttt ggc ttg ttt gac aaa gat ggt gat ggg tgc att act gtg 158
Glu Ala Phe Gly Leu Phe Asp Lys Asp Gly Asp Gly Cys Ile Thr Val
 15                  20                  25                  30

gac gaa ttt gtc acg gtt atc cgg tca ttg gtt cag aac ccc aca gaa 206
Asp Glu Phe Val Thr Val Ile Arg Ser Leu Val Gln Asn Pro Thr Glu
                 35                  40                  45

gaa gag ctc caa gac atg ata aac gag gta gat gca gat ggt aat gga 254
Glu Glu Leu Gln Asp Met Ile Asn Glu Val Asp Ala Asp Gly Asn Gly
             50                  55                  60

acc att gaa ttt gtt gag ttt ttg aac tta atg gcc aag aaa atg aag 302
Thr Ile Glu Phe Val Glu Phe Leu Asn Leu Met Ala Lys Lys Met Lys
         65                  70                  75

gaa act gat gaa gag gaa gat ctc aaa gag gct ttc aag gtg ttt gac 350
Glu Thr Asp Glu Glu Glu Asp Leu Lys Glu Ala Phe Lys Val Phe Asp
     80                  85                  90

aag gat caa aat ggc tac att tca gca agt gag ttg aga cac gtt atg 398
Lys Asp Gln Asn Gly Tyr Ile Ser Ala Ser Glu Leu Arg His Val Met
 95                  100                 105                 110

atc aat ctg ggt gaa aaa cta act gat gag gag gtg gag cag atg att 446
Ile Asn Leu Gly Glu Lys Leu Thr Asp Glu Glu Val Glu Gln Met Ile
                115                 120                 125

gaa gaa gca gat ttg gat ggt gat ggt caa gtt aat tat gat gaa ttt 494
Glu Glu Ala Asp Leu Asp Gly Asp Gly Gln Val Asn Tyr Asp Glu Phe
```

130 135 140

gtc aag atg atg atg act att gga tgaaattttc aaacaacaaa tttattgttc 548
Val Lys Met Met Met Thr Ile Gly
145 150

gaattcgatc atttgggccc caatctcatt cactcgcatg aaataataag tcttccaatc 608

aatttttggt gtattttact tgtagcacat gatatgaatg accaatgtat tagagtgaca 668

tctcagccaa tttgatttgt tcaaactcca acccataatt attctttcag ttttagttta 728

gcattcctta tgttttaaaa ttaattagag ggaatttatt caagaacttc tgaaattaga 788

ctctttttg aaagattaat gtatgctaca atttattctt tctcttgtga acatggtatt 848

gctacaattt atttatatca ttcagaaaaa taaaaaacag cacaagaatt aaacaaaaaa 908

aaaaaaaa 916

<210> 4
<211> 150
<212> PRT
<213> Unknown Organism

<400> 4

Met Ala Asp Val Leu Ser Glu Glu Gln Ile Ser Glu Ile Lys Glu Ala
1 5 10 15

Phe Gly Leu Phe Asp Lys Asp Gly Asp Gly Cys Ile Thr Val Asp Glu
20 25 30

Phe Val Thr Val Ile Arg Ser Leu Val Gln Asn Pro Thr Glu Glu Glu
35 40 45

Leu Gln Asp Met Ile Asn Glu Val Asp Ala Asp Gly Asn Gly Thr Ile
50 55 60

Glu Phe Val Glu Phe Leu Asn Leu Met Ala Lys Lys Met Lys Glu Thr
65 70 75 80

Asp Glu Glu Glu Asp Leu Lys Glu Ala Phe Lys Val Phe Asp Lys Asp
85 90 95

Gln Asn Gly Tyr Ile Ser Ala Ser Glu Leu Arg His Val Met Ile Asn
100 105 110

```
Leu Gly Glu Lys Leu Thr Asp Glu Glu Val Glu Gln Met Ile Glu Glu
        115                 120                 125

Ala Asp Leu Asp Gly Asp Gly Gln Val Asn Tyr Asp Glu Phe Val Lys
    130                 135                 140

Met Met Met Thr Ile Gly
145                 150
```

Claims

1. A method for enhancing multiple disease resistance of a higher plant to attack by one or more plant pathogens wherein cells of said plant, or the progeny of said cells, are transformed with and express a gene encoding either soybean calmodulin 4 (ScaM4) or soybean calmodutin 5 (ScaM5) which have sequences as shown in SEQ ID No.: 1 and SEQ.ID. No.: 2, respectively.

2. The method of claim 1 wherein the plant is of a dicot species.

3. The method of claim 2, wherein the plant is tobacco.

4. The method of any claim 1 to 3, wherein cells of said plant, or the progeny of said cells, are transformed with a binary vector comprising a nucleotide sequence encoding ScaM4 or ScaM5 (SEQ ID NO.: 1 and SEQ ID NO.: 2, respectively).

5. The method of claim 4 wherein the binary ScaM4 or ScaM5 vector comprise regulatory elements for the expression in plant cells.

6. The method of claim 5 wherein said regulatory elements are the 35 S promoter from cauliflower mosaic virus, a translation enhancer sequence, and the nopaline synthase transcription termination sequence.

7. The method of claim 6, wherein said binary vector is pLES9804 or pLES9805 (KCTC Accession No. 0545BP and KCTC Accession No. 0546BP, respectively).

8. Use of a transgenic plant obtained by a method as claimed in any preceding claim 1 to 7 for reducing the amount of fungicides applied to control the decay and for obtaining a higher crop yield.

Patentansprüche

1. Verfahren zur Erhöhung der Resistenz einer höheren Pflanze gegen multiple Krankheiten bei einer Attacke von ein oder mehreren Pflanzenpathogenen, wobei Zellen der Pflanze oder deren Nachkommen transformiert sind und ein Gen exprimieren, das entweder Soja-Calmodulin-4 (ScaM4) oder Soja-Calmodulin-5 (ScaM5) kodiert, deren Sequenzen wie in SEQ ID NO: 1 bzw. SEQ ID NO: 2 sind.

2. Verfahren nach Anspruch 1, wobei die Pflanze eine dicoide Spezies ist.

3. Verfahren nach Anspruch 2, wobei die Pflanze Tabak ist.

4. Verfahren nach irgendeinem Anspruch 1 bis 3, wobei Zellen der Pflanze oder deren Nachkommen transformiert sind mit einem binären Vektor, der eine Nukleotidsequenz aufweist, die ScaM4 oder ScaM5 (SEQ ID Nr. 1 bzw. SEQ ID Nr. 2) kodiert.

5. Verfahren nach Anspruch 4, wobei der binäre ScaM4- oder ScaM5-Vektor regulatorische Elemente für die Expression in Pflanzenzellen aufweist.

6. Verfahren nach Anspruch 5, wobei die regulatorischen Elemente sind: der 35-S-Promoter des Blumenkohl-Mosaikvirus, eine Translations-Enhancersequenz und die Terminationssequenz der Nopalinsynthasetranskription.

7. Verfahren nach Anspruch 6, wobei der binäre Vektor pLES9804 oder pLES9805 (KCTC-Zugangsnummern 0545BP bzw. 0546BP) ist.

8. Verwendung einer transgenen Pflanze, die durch ein Verfahren nach irgendeinem der vorhergehenden Ansprüche 1 bis 7 gewonnen wurde, zur Reduktion der Menge an Fungiziden, die zur Bekämpfung des Verfalls ausgebracht werden, und zur Erreichung eines höheren Ernteertrags.

Revendications

1. Procédé pour l'augmentation de la résistance aux maladies multiples d'une plante supérieure suite à une attaque par un ou plusieurs agents pathogènes de la plante, dans lequel des cellules de ladite plante, ou la lignée desdites cellules, sont transformées avec et expriment un gène encodant la calmoduline 4 du soja (ScaM4) ou la calmoduline 5 du soja (ScaM5) qui ont des séquences comme représenté dans SEQ ID NO : 1 et SEQ ID NO : 2, respectivement.

2. Procédé selon la revendication 1, dans lequel la plante est d'une espèce de dicotylédone.

3. Procédé selon la revendication 2, dans lequel la plante est le tabac.

4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel des cellules de ladite plante, ou la lignée desdites cellules, sont transformées avec un vecteur binaire comprenant une séquence de nucléotides encodant ScaM4 ou ScaM5 (SEQ ID NO : 1 et SEQ ID NO : 2, respectivement).

5. Procédé selon la revendication 4, dans lequel le vecteur binaire de ScaM4 ou de ScaM5 comprend des éléments régulateurs pour l'expression dans les cellules de la plante.

6. Procédé selon la revendication 5, dans lequel lesdits éléments régulateurs sont le promoteur 35 S du virus mosaïque du chou-fleur, une séquence activatrice de la traduction, et la séquence de terminaison de la transcription de la nopaline synthase.

7. Procédé selon la revendication 6, dans lequel ledit vecteur binaire est pLES9804 ou pLES9805 (numéro d'accès KCTC 0545BP et numéro d'accès KCTC 0546BP, respectivement).

8. Utilisation d'une plante transgénique obtenue par un procédé selon l'une quelconque des revendications précédentes 1 à 7, pour la réduction de la quantité de fongicides appliquée pour contrôler la décomposition et pour l'obtention d'un rendement des cultures supérieur.

FIG. 1

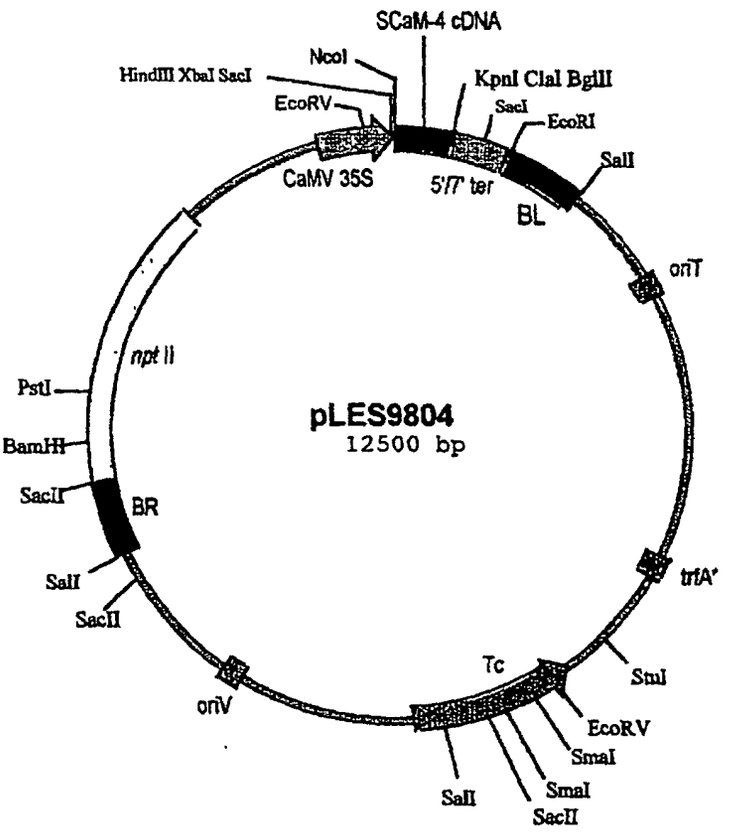
SCaM-4 cDNA
NcoI
HindIII XbaI SacI
EcoRV
KpnI ClaI BglII
SacI
EcoRI
SalI
CaMV 35S
5'T' ter
BL
oriT
npt II
PstI
BamHI
SacII
BR
SalI
SacII
oriV
Tc
StuI
EcoRV
SmaI
SmaI
SacII
SalI
trfA'
pLES9804
12500 bp

FIG. 2

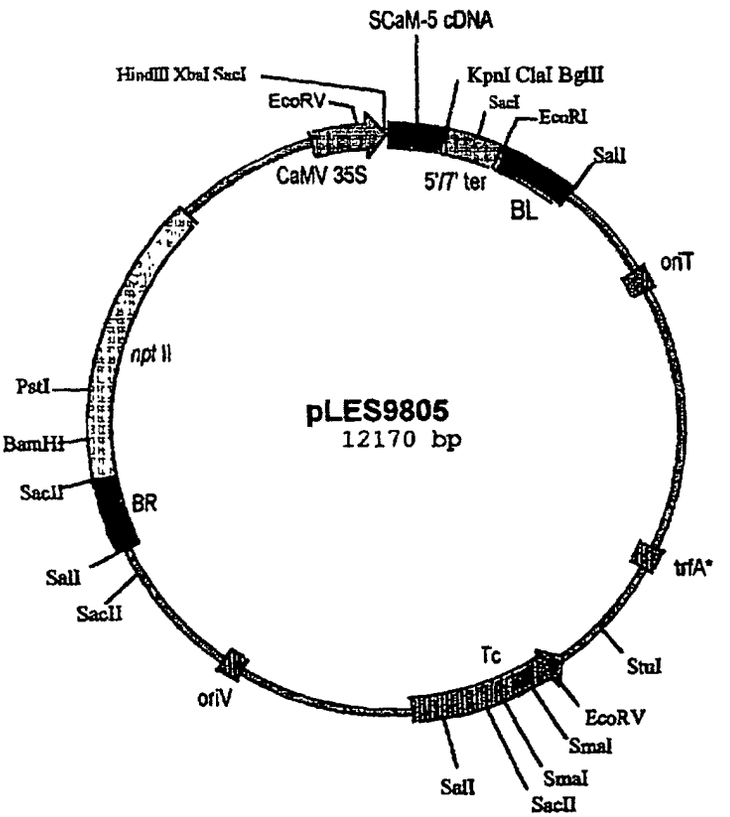
SCaM-5 cDNA
HindIII XbaI SacI
EcoRV
KpnI ClaI BglII
SacI
EcoRI
SalI
CaMV 35S
5'/7' ter
BL
oriT
npt II
PstI
BamHI
SacII
BR
SalI
SacII
oriV
Tc
StuI
EcoRV
SmaI
SalI
SmaI
SacII
trfA*
pLES9805
12170 bp

FIG. 3

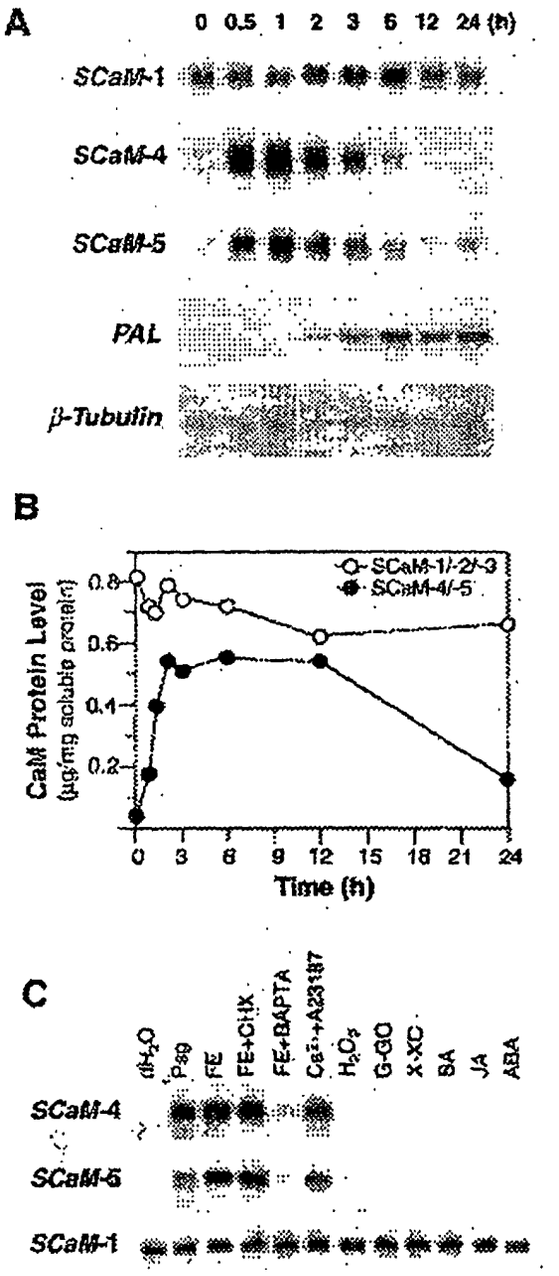
A
0 0.5 1 2 3 5 12 24 (h)
SCaM-1
SCaM-4
SCaM-5
PAL
β-Tubulin
B
SCaM-1/-2/-3
SCaM-4/-5
CaM Protein Level
(μg/mg soluble protein)
0.8
0.6
0.4
0.2
0 3 6 9 12 15 18 21 24
Time (h)
C
dH2O
Pag
FE
FE+CHX
FE+BAPTA
Ca2+A23187
H2O2
G-GO
X-XC
BA
JA
ABA
SCaM-4
SCaM-5
SCaM-1

FIG. 4

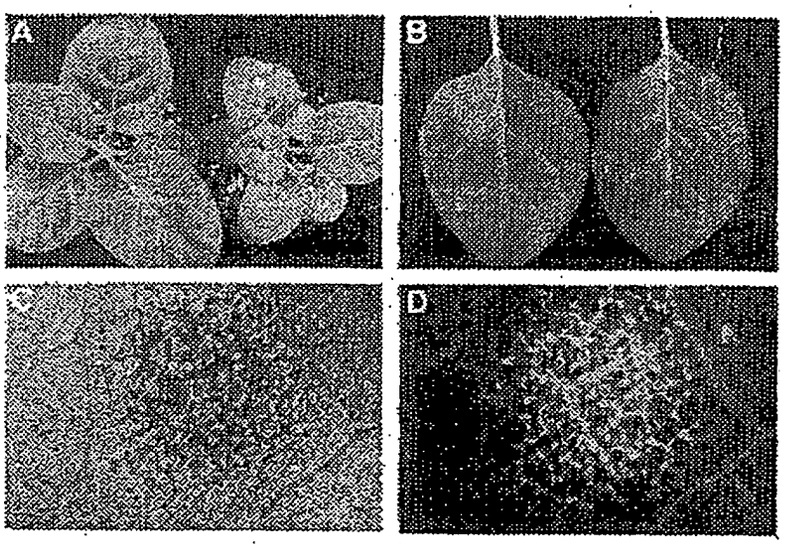

FIG. 5

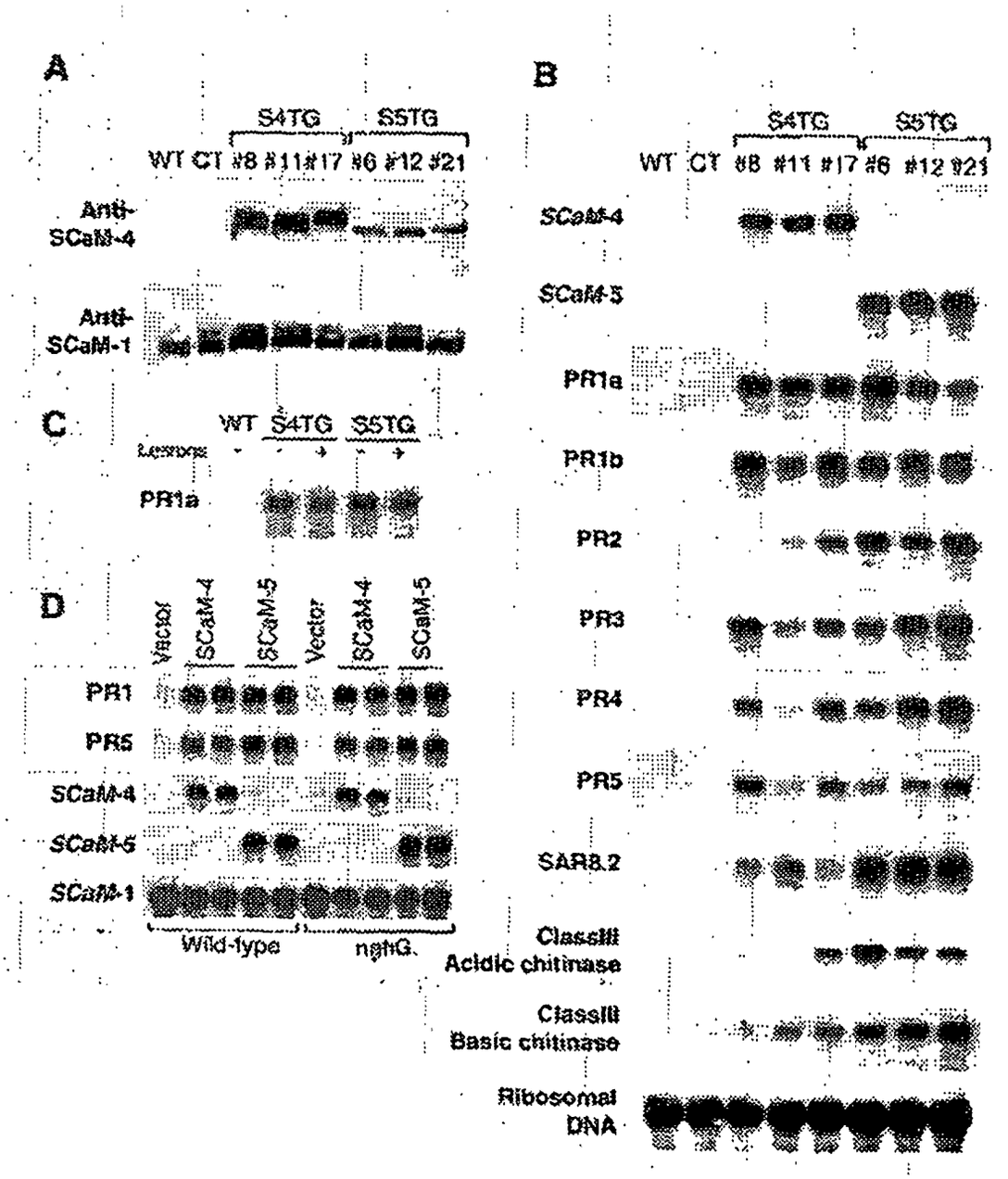
A
S4TG
S5TG
WT CT #8 #11 #17 #6 #12 #21
Anti-SCaM-4
Anti-SCaM-1
C
WT S4TG S5TG
Lesions
PR1a
D
Vector
SCaM-4
SCaM-5
Vector
SCaM-4
SCaM-5
PR1
PR5
SCaM-4
SCaM-5
SCaM-1
Wild-type
ngnG
B
S4TG
S5TG
WT CT #8 #11 #17 #6 #12 #21
SCaM-4
SCaM-5
PR1a
PR1b
PR2
PR3
PR4
PR5
SAR8.2
ClassIII Acidic chitinase
ClassII Basic chitinase
Ribosomal DNA

FIG. 6